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Risk of Development of In Vitro Resistance to Amoxicillin, Clarithromycin, and Metronidazole in Helicobacter pylori

MIKAEL SÖRBERG,¹ HÅKAN HANBERGER, ² MAUD NILSSON,³ ANDERS BJÖRKMAN,¹ AND LENNART E. NILSSON³

Department of Infectious Diseases, Danderyd Hospital, S-182 88 Danderyd,¹ and Infectious Diseases and Department of Clinical Microbiology, ² University Hospital, S-581 85 Linköping, Sweden

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We have studied initial killing, morphological alterations, the frequency of occurrence, and the selective growth of resistant subpopulations of Helicobacter pylori during exposure to amoxicillin, clarithromycin, or metronidazole by bioluminescence assay of intracellular ATP levels, microscopy, and a viable count assay. We found an induction of spheroplasts and a decrease in intracellular ATP levels after 21 h of exposure to high concentrations of amoxicillin. During clarithromycin exposure the onset of a decrease in intracellular ATP levels started after prolonged incubation, and with the highest concentration of clarithromycin an induction of coccoid forms was seen after 68 h. Metronidazole exposure resulted in the strongest initial decrease in intracellular ATP levels, and coccoid forms were seen after 21 h of exposure to high concentrations of metronidazole. Amoxicillin caused a low-level increase in resistant subpopulations, which indicates a need for surveillance of the amoxicillin susceptibility of H. pylori in order to detect decreasing susceptibility. No increase in the numbers of resistant subpopulations was demonstrated during clarithromycin exposure. Metronidazole selected resistant subpopulations, which caused high-level resistance in H. pylori.

Helicobacter pylori infection is a principal cause of chronic gastritis type B (13) and is associated with gastric cancer (19, 20, 33). Eradication of H. pylori prevents relapse of duodenal ulcer, and treatment of this infection has now become standard for patients with peptic ulcer disease (22, 37). The regimen most widely used today to eradicate H. pylori is combination therapy with two antibiotics and bismuth (17, 27) or an acid pump inhibitor (3, 22). A major reason for H. pylori eradication failure is resistance to metronidazole (4, 5, 9, 17, 27, 29, 34, 36) or clarithromycin (11, 44). In contrast, H. pylori does not appear to develop resistance to amoxicillin (16). The prevalence of primary metronidazole resistance varies between 7 and 90% (2, 9, 14, 36), with the highest prevalences occurring in people from developing countries (2, 9, 14), after previous metronidazole ingestion (2, 14, 36), and in women (2, 14, 36). The prevalence of primary clarithromycin resistance is between 4 and 7% (12, 16, 45). The development of resistance during therapy has been observed for both metronidazole (35, 36, 46) and clarithromycin (11, 26, 43).

The aim of this study was to evaluate the initial killing, morphological alterations, and the frequency of occurrence and the selective growth of resistant subpopulations of H. pylori during exposure to amoxicillin, clarithromycin, or metronidazole.

MATERIALS AND METHODS

Bacterial strain. H. pylori NCTC 11637 was used in the present study.

Antibiotics. Amoxicillin and metronidazole were purchased from Sigma Chemical Co., St. Louis, Mo. Clarithromycin was kindly provided by Abbott Laboratories, Chicago, Ill.

Growth medium. Mueller-Hinton broth (MHb; Gibco Limited, Renfrewshire, Scotland) supplemented with 50 mg of Ca²⁺ per liter, 25 mg of Mg²⁺ per liter, and 1% fetal calf serum was used as the growth medium.

Bioluminescence assay of intracellular ATP levels. A 100-μl sample from the bacterial culture was incubated at 37°C for 10 min with 100 μl of the ATP-hydrolyzing enzyme apyrase, purified grade I (Sigma Chemical Co.) in supplemented MHb, to eliminate extracellular ATP. A 50-μl sample of the apyrase-treated sample was pipetted into 500 μl of boiling 0.1 M Tris buffer (pH 7.75) containing 2 mM EDTA to release the intracellular ATP and inactivate the apyrase. After being heated for 90 s, the extracts were cooled before the intracellular ATP levels were assayed. This extraction was performed in an LKB Biocal 2030 incubator (LKB Products, Bromma, Sweden). Luciferase reagent (100 μl) was added to 550 μl of each cooled extract, and the light intensity was measured in a 1250 Luminometer (LKB Wallac, Turku, Finland) and was recorded on a 1250 Display (LKB Wallac). An ATP-monitoring reagent (Bio Orbit, Turku, Finland) was used in the assay of ATP levels. The ATP levels in the samples were calculated by using the results of assays of standard amounts of ATP as a reference. A correction was made for background luminescence.

Extracts to which known amounts of ATP were added were used as internal standards in order to correct for inhibition of the luciferase reaction by the reagents. The coefficient of variation for the bioluminescence assay has been shown to vary between 1.7 and 6.5% (41).

Monitoring of bacterial growth during antibiotic exposure in broth. The bacterial numbers were determined by the bioluminescence assay of bacterial ATP levels. As indicated by Thore et al. (42) and Molin et al. (30), 10⁻⁶ M ATP corresponded to approximately 10⁹ bacteria/ml. From a culture with bacteria in the log phase diluted to 10⁻⁶ CFU/ml, 5 ml was transferred to 50-ml Erlenmeyer flasks containing 50 μl of antibiotics at different concentrations. The concentrations tested were as follows: amoxicillin, 0.0005 to 0.25 μg/ml; clarithromycin, 0.0005 to 0.25 μg/ml; and metronidazole, 0.06 to 32 μg/ml. Samples were taken daily for bioluminescence assay of intracellular bacterial ATP levels. The flasks were incubated at 37°C under microaerobic conditions (5% oxygen, 10% carbon dioxide, 85% nitrogen) in an incubator box (ASSAB with CO₂ and O₂ regulator; Kebo Biomed, Spånga, Sweden). The experiments were repeated three times.

Morphology. The bacteria were exposed to amoxicillin, clarithromycin, and metronidazole and were studied by light microscopy at a magnification of ×1,250 after being stained with acridine orange (28). The sensitivity of the staining method is 10⁶ CFU/ml (28).

Population analysis. Population analyses were performed with control cultures and regrowing cultures exposed to metronidazole or amoxicillin. Population analyses were also performed with cultures that grew in the presence of the highest concentration during clarithromycin exposure. The contents of the flasks were thoroughly mixed, and 0.1-ml portions were removed and diluted serially in 0.9-ml aliquots of phosphate-buffered saline. A total of 50 μl from each dilution was spread onto paper disc method (PDM) agar plus 5% defibrinated horse blood (AB Biodisk, Solna, Sweden) containing different concentrations of amoxicillin (0.0005 to 0.25 μg/ml), clarithromycin (0.0005 to 0.25 μg/ml), and metronidazole (0.06 to 32 μg/ml). The drops were allowed to dry at room temperature.

The plates were incubated for 5 days at 37°C under microaerobic conditions, and the colonies were then counted. The frequencies of occurrence of variants resistant to different concentrations of amoxicillin, clarithromycin, and metronidazole were calculated by dividing the number of colonies on plates with antibiotic.
by the number of colonies on plates without antibiotic. The experiments were repeated three times. Ten passages of the metronidazole-resistant cultures were done in drug-free MHB. Susceptibility testing on agar plates was done with passaged cultures in antibiotic-free broth by the E-test (AB Biodisk).

**Determination of antibiotic concentrations in H. pylori cultures.** Samples from *H. pylori* cultures exposed to 0.25 µg of amoxicillin per ml, 0.25 µg of clarithromycin per ml, or 32 µg of metronidazole per ml were taken after 0, 21, 46, 68, 96, 118, 142, 166, 191, 214, and 267 h incubation at 37°C under microaerobic conditions. The samples were put in wells of a PDM agar (AB Biodisk) tray with *Micrococcus luteus* ATCC 9341 for determination of amoxicillin and clarithromycin concentrations and in wells of a PDM agar plus 5% defibrinated horse blood (AB Biodisk, Solna, Sweden) tray with *Clostridium perfringens* ATCC 13124 for determination of metronidazole concentration. The trays with *M. luteus* were incubated overnight under aerobic conditions, and the trays with *C. perfringens* were incubated overnight under anaerobic conditions. The resulting inhibition zones surrounding the wells were measured and were compared with those obtained by linear regression analysis with standard concentrations of drugs.

**RESULTS**

**Growth and morphology of H. pylori during exposure to amoxicillin.** Growth of the cultures with an inoculum of 1.9 × 10^−8 M ATP was monitored, and three growth patterns were seen. Intracellular ATP levels increased in the cultures exposed to low concentrations of amoxicillin (≤0.004 µg/ml) (Fig. 1). There was an initial growth inhibition in the cultures exposed to 0.008 µg of amoxicillin per ml (Fig. 1). The cultures exposed to ≥0.015 µg of amoxicillin per ml showed an initial decrease in intracellular ATP levels after 46 h (Fig. 1). Microscopy showed spheroplasts in cultures in which a decrease in intracellular ATP levels occurred. After 118 h a few bacillary forms were seen, and the numbers of these forms increased when the intracellular ATP level in these cultures increased. There was no decrease in the amoxicillin concentration in the broth cultures containing 0.008 and 0.015 µg of amoxicillin per ml when regrowth occurred. In the culture exposed to 0.03 µg of amoxicillin per ml, intracellular ATP levels increased after 191 h, and microscopy showed a mixed population of bacillar forms, spheroplasts, and coccoid cells. In this culture there was a reduction in the amoxicillin concentration, and at 191 h, when the culture regrew, only 50% of the initial amoxicillin concentration was left in the broth. At concentrations above 0.03 µg of amoxicillin per ml there was no regrowth.

**Population analyses of cultures exposed to amoxicillin.** Population analyses were performed with the unexposed cultures and cultures exposed to amoxicillin at concentrations of 0.008, 0.015, and 0.03 µg/ml. The bacteria in the unexposed cultures were resistant to up to 0.008 µg/ml (Fig. 2). At higher concentrations on agar plates there was a reduction in the frequency of resistant variants for the unexposed broth cultures (Fig. 2). The cultures exposed to 0.008 and 0.015 µg of amoxicillin per ml in broth were resistant to up to 0.015 µg/ml, and the frequency of resistant variants exposed to amoxicillin at 0.03 µg/ml was 10^{-1} (Fig. 2). The frequency of resistant variants in the cultures exposed to 0.03 µg of amoxicillin per ml was lower than that for the unexposed cultures (Fig. 2). Population analyses were performed with the control cultures on two different occasions with an interval of 3 days, and no reduction in the amoxicillin concentrations on the agar plates was found.

**Growth and morphology of H. pylori during exposure to clarithromycin.** Growth of the cultures with an inoculum of 2.3 × 10^−8 M ATP was monitored. Intracellular ATP levels increased in the cultures exposed to low concentrations of clarithromycin (≤0.06 µg/ml) (Fig. 3). There was a concentration-dependent inhibition of the increase in intracellular ATP levels, and after prolonged incubation there was a concentration-dependent decrease in intracellular ATP levels in the cultures (Fig. 3). Microscopy showed bacillary forms in the growing cultures, and a conversion from bacillary to coccoid forms was seen when the intracellular ATP levels decreased. No cultures showed regrowth. The clarithromycin concentrations in broth were stable throughout the experiment.

**Population analyses of cultures exposed to clarithromycin.** Population analyses were performed with the unexposed cultures and cultures exposed to clarithromycin at concentrations of 0.015 and 0.008 µg/ml. The unexposed cultures and the cultures exposed to 0.008 µg of clarithromycin per ml were resistant to up to 0.015 µg/ml (Fig. 4). At higher concentrations there was a reduction in the frequency of resistant variants to 10^{-1} after exposure to clarithromycin at 0.03 µg/ml (Fig. 4). For cultures exposed to 0.015 µg of clarithromycin per ml the frequency of resistant variants was lower after exposure
to clarithromycin at 0.015 and 0.03 \( \mu g/ml \) than that for the unexposed cultures and the cultures exposed to 0.008 \( \mu g/ml \) (Fig. 4). Population analyses were performed with the control cultures on two different occasions with an interval of 3 days, and no reduction in the clarithromycin concentration in the agar plates was found.

**Growth and morphology of** *H. pylori** during exposure to metronidazole.* Growth of the cultures with an inoculum of \( 1.9 \times 10^{-8} \) M ATP was monitored, and two growth patterns were seen. Intracellular ATP levels increased in the cultures exposed to low concentrations of metronidazole (\( \leq 0.5 \) \( \mu g/ml \)) (Fig. 5). The cultures exposed to 1 to 32 \( \mu g/ml \) of metronidazole showed an initial decrease in intracellular ATP levels (Fig. 5), and microscopy showed a conversion from bacillary to coccoid forms after 21 h. When the intracellular ATP levels increased in the cultures exposed to 1 to 4 \( \mu g/ml \) of metronidazole, a change in morphology was seen by microscopy, from coccoid forms to bacillary forms. In cultures with concentrations above 4 \( \mu g/ml \) there was no regrowth (Fig. 5). The metronidazole concentrations in broth were stable throughout the experiment.

**Population analyses of cultures exposed to metronidazole.** Population analyses were performed with the unexposed cultures and cultures exposed to metronidazole at concentrations of 1 to 4 \( \mu g/ml \). The bacteria in the unexposed cultures were resistant to up to 0.25 \( \mu g/ml \) (Fig. 6). With higher concentrations there was a reduction in the frequency of resistant variants for the unexposed cultures (Fig. 6). For the cultures exposed...
posed to 1 to 4 μg of metronidazole per ml, resistant variants were resistant to up to 32 μg/ml (Fig. 6). The selection of resistant variants was concentration dependent, and after exposure to the highest concentration (4 μg of metronidazole per ml) all bacteria in the population were resistant to metronidazole at 32 μg/ml (Fig. 6). Population analyses were performed with the control cultures on three different occasions with a total interval of 13 days, and no reduction in the metronidazole concentrations on the agar plates was found (Fig. 6). The resistance remained stable through 10 passages in MHB for all cultures in which metronidazole resistance developed.

**DISCUSSION**

This study showed an initial decrease in intracellular ATP levels during exposure of *H. pylori* to high concentrations of amoxicillin (Fig. 1), and this bactericidal effect of amoxicillin is in agreement with the effect found in a study by Berry et al. (6). During exposure of *H. pylori* to amoxicillin, microscopy showed spheroplasts after 21 h, which is in accordance with the findings of a study by Nilius et al. (32) and a previous study by us (40), in which we found a concentration-dependent induction of spheroplasts after only 2 h. Other investigators have reported a morphologic conversion of *H. pylori* during exposure to...
amoxicillin but have not distinguished between coccoid forms and spheroplasts (6, 7). Armstrong et al. (1) reported central clearing and vesiculation of *H. pylori* after a 24-h exposure to amoxicillin but did not discuss these findings in terms of coccoid forms or spheroplasts. No reports on the clinical significance of amoxicillin resistance in *H. pylori* have been published, and Glupczynski et al. (16) found an unchanged susceptibility of *H. pylori* to amoxicillin over a 5-year period. We found a small increase in the numbers of resistant subpopulations in all except one of the regrowing cultures exposed to amoxicillin. In one culture (containing 0.03 µg of amoxicillin per ml), regrowth occurred after 191 h due to a decrease in the concentration of amoxicillin in the broth (Fig. 2). Our results are in agreement with those of Haas et al. (18), who found increased amoxicillin MICs during exposure to amoxicillin in several passages. Our results and those of Haas et al. (18) indicate that there is a need for surveillance of the amoxicillin susceptibility of *H. pylori* in order to detect decreasing levels of susceptibility.

After a prolonged incubation, clarithromycin exposure resulted in a concentration-dependent decrease in intracellular ATP levels in *H. pylori* cultures (Fig. 3). Similar results were found by Flamm et al. (15), who demonstrated a bactericidal effect of clarithromycin on *H. pylori* after 8 h of exposure to clarithromycin. In an earlier study (40) we could not find any bactericidal effect after 5 h of exposure of *H. pylori* to clarithromycin, but this was probably due to a shorter exposure time. A conversion from bacillary to coccoid forms was seen in this study after 68 h of exposure of *H. pylori* to the highest concentration of clarithromycin. After the same exposure time, Nilius et al. (32) found an induction of coccoid forms during exposure of *H. pylori* to erythromycin. We found no regrowth or increase in the numbers of resistant subpopulations of *H. pylori* during clarithromycin exposure (Fig. 4). Haas et al. (18) found variation among different strains, with increasing MICs for *H. pylori* during exposure to erythromycin. This variation among different strains might explain why we did not find resistant subpopulations of the strain used in our study.

The strongest initial decrease in intracellular ATP levels was seen during exposure of *H. pylori* to metronidazole (Fig. 5), and this bactericidal effect of metronidazole on *H. pylori* has also been demonstrated by Armstrong et al. (1). We found a conversion from bacillary to coccoid forms after 21 h in the cultures in which a decrease in intracellular ATP levels occurred. This is similar to the results of a study by Armstrong et al. (1), who reported a conversion to coccoid forms after 48 h of exposure of *H. pylori* to metronidazole. The mechanisms for metronidazole resistance are probably a decreased ability of metronidazole-resistant strains to achieve a sufficiently low redox potential under microaerobic conditions for the necessary reduction of metronidazole and that during short periods of anaerobic conditions these strains manage to reduce and store sufficient amounts of metronidazole so as to appear fully susceptible after subsequent incubation under microaerobic conditions (8, 38, 48). This leads to a slower uptake of metronidazole by resistant strains of *H. pylori* than by sensitive strains, which has been reported by several investigators (25, 31, 38). We found selection and regrowth of resistant subpopulations of *H. pylori* in all regrowing cultures during exposure to metronidazole (Fig. 6). During this regrowth a change in morphology from coccoid to bacillary forms was seen by microscopy. This change is probably due to a low frequency of occurrence of resistant subpopulations rather than a conversion from coccoid to bacillary forms. This caused a decreased susceptibility to metronidazole, which is in agreement with the findings of Haas et al. (18), who reported an increase in the MIC of metronidazole for *H. pylori* after several passages during exposure to metronidazole. When evaluating the development of resistance to metronidazole during treatment, it is important to study whether there is a selection of spontaneous resistant variants of the infecting strain or whether there is reinfection with an exogenous strain (23, 35). The metronidazole resistance in our study was stable during 10 passages, which is in agreement with the findings of a study by Haas et al. (18), but there have also been reports of unstable metronidazole resistance. Zwet et al. (48) found that metronidazole resistance induced in vitro was reversed in 30% of the isolates by further culture on antibiotic-free plates. The different results concerning the stability of metronidazole resistance might be explained by methodological differences such as the use of...
different inocula (21) and by the use of different incubation conditions (8, 38, 47). When studying the morphology of H. pylori it is important to differentiate between coccoïds (1, 6) and spheroplasts (32). In our earlier study we found a rapid induction of spheroplasts during exposure of H. pylori to amoxicillin (40), while the rate of conversion to coccoïd forms during exposure to clarithromycin and metronidazole in this study was slower. The spheroplasts are larger than the coccoïd forms seen by microscopy (unpublished results). The coccoïd forms changed color, from orange to green, during prolonged exposure to clarithromycin and metronidazole when acridine orange staining was used (unpublished results). A cell containing more RNA than DNA stains orange (unpublished results). A cell containing more RNA than DNA degraded but still retains its DNA stains green (24). The degradation of RNA has been correlated with a loss of viability (10). The spheroplasts stained orange (unpublished results), and we found in an earlier study (40) that they reverted to bacillary forms. In another previous study (39) we found a low ATP level in the coccoïd cell during prolonged incubation of H. pylori, but we were not able to demonstrate a conversion to bacillary forms. In conclusion, we found an induction of spheroplasts and a decrease in intracellular ATP levels after 21 h of exposure of H. pylori to high concentrations of amoxicillin. During clarithromycin exposure the onset of decrease in intracellular ATP levels started after prolonged incubation, and with the highest concentration of clarithromycin an induction of coccoïd forms was seen after 68 h. Metronidazole exposure resulted in the strongest initial decrease in intracellular ATP levels, and coccoïd forms were seen after 21 h of exposure to high concentrations of metronidazole. Amoxicillin caused a low-level increase in the numbers of resistant subpopulations, which indicates that there is a need for surveillance of the amoxicillin susceptibility of H. pylori in order to detect decreasing susceptibility. No increase in resistant subpopulations was demonstrated during clarithromycin exposure. Metronidazole selected resistant subpopulations, which caused high-level resistance in H. pylori.

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